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Structural Identification of Dehydrotriferulic and Dehydrotetraferulic Acids Isolated from Insoluble Maize Bran Fiber

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Two new dehydrotriferulic acids and two dehydrotetraferulic acids were isolated from saponified maize bran insoluble fiber using size exclusion chromatography on Bio-Beads S-X3 followed by Sephadex LH-20 chromatography and semipreparative phenyl-hexyl reversed phase high-performance liquid chromatography. On the basis of UV spectroscopy, mass spectrometry, and one- and two-dimensional NMR experiments, the structures were identified as 8-5(noncyclic)/5-5-dehydrotriferulic acid, 8-8(tetrahydrofuran)/5-5-dehydrotriferulic acid, and 4-*O*-8/5-5/8-*O*-4-dehydrotetraferulic acid. The second tetramer was tentatively identified as 4-*O*-8/5-5/8-5(noncyclic)-dehydrotetraferulic acid. Compounds containing an 8-5(noncyclic)-coupled dimeric unit probably do not exist in planta but are formed from their phenylcoumaran precursors containing an 8-5(cyclic)-coupled dimeric unit during saponification. The presented dehydrotrimers are the first dehydrotriferulates that do not contain an 8-*O*-4-coupled dimeric unit. The ferulate dehydrotetramers that are reported for the first time are presumed, like the dimers and trimers, to cross-link polysaccharides in the plant. Because both tetramers contain a 5-5/8-*O*-4-dehydrotriferulate moiety, the predominant dehydrotrimer in maize bran, it is not possible to deduce whether tetramers are formed by coupling of a fourth unit to a preformed dehydrotriferulate or by 5-5-coupling of preformed 8-*O*-4- and 8-5-dehydrodiferulates. Nevertheless, such compounds document expanded roles for ferulates in cross-linking polysaccharides in plant cell walls.

KEYWORDS: Cell wall cross-linking; tetraferulic acid; tetraferulate; dehydrotetramer; triferulic acid; triferulate; dehydrotrimer; ferulic acid; ferulate; arabinoxylans; dietary fiber; *Zea mays* L.; radical coupling; NMR

INTRODUCTION

Cross-linking of plant cell wall polymers has a marked influence on the properties of the cell wall itself and on the use of cell walls or cell wall components as food or feed. Hydroxycinnamates are able to cross-link polysaccharides with each other, with lignin, and probably with proteins (*1*, *2*). Ferulates and their corresponding dimers especially have been extensively studied in the past. In graminaceous plants, ferulic acid acylates the C5−OH of α-L-arabinosyl moieties of arabinoxylans (*3*−*5*), and in some plants belonging to the broadly circumscribed family of Amaranthaceae, ferulic acid is linked to pectic arabinans and galactans (*3*, *6*, *7*). Ferulate dimerization is achieved by a photochemical mechanism leading to cyclobutane dimers (*8*) and, more importantly, by radical coupling mechanisms (*9*, *10*) with dehydrodiferulates as reaction products. Following saponification, radically coupled dehydrodiferulic acids have been routinely proven in a variety of plant materials in the past. Ferulate dimers have been considered responsible for phenomena such as terminating the expansion of cell wall growth (*11*) and cell wall stiffening (*12*), thermal stability of cell wall adhesion influencing the texture of cooked fruits and vegetables (*13*), gelling properties of pectins and arabinoxylans (*14*, *15*), insolubility of cereal dietary fibers (*16*), and limited cell wall degradability by ruminants (*17*).

From studies on maize suspension cultures, it was speculated that higher oligomers than dehydrodiferulates exist in the plant (*18*). Recently, this theory has been proven by the isolation and structural elucidation of five dehydrotrimers of ferulic acid from maize bran (*19*−*22*). In addition to the probably predominant 5-5/8-*O*-4-dehydrotriferulic acid, 8-*O*-4/8-*O*-4-, 8-8(cyclic)/8-*O*-4-, 8-*O*-4/8-5(noncyclic)-, and 5-5/8-*O*-4(H_2O)-dehydrotriferulic acids were isolated and structurally characterized.

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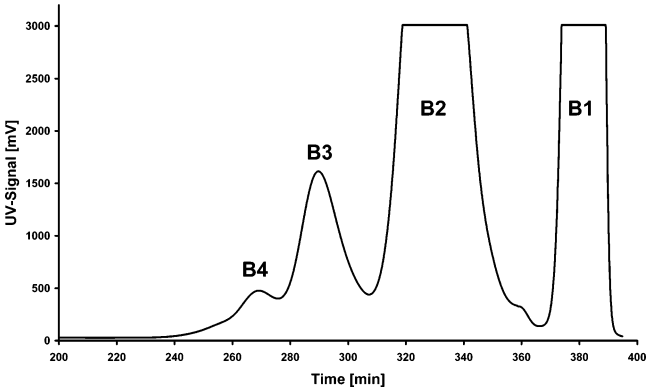

Figure 1. Size-exclusion chromatogram on Bio-Beads S-X3 of a purified, alkaline hydrolysate of maize bran fiber. Fractions: B1, primary phenolic monomers; B2, phenolic dimers; B3, phenolic trimers; and B4, phenolic tetramers. Note that monomers B1 elute faster due to an enhanced flow rate.

Defined structures of higher oligomers than trimers have not been reported to date.

In this study, we isolated and structurally characterized two dehydrotriferulic acids not containing 8-*O*-4-coupled diferulate moieties and two dehydrotetraferulic acids representing a class described for the first time. Possible formation pathways are discussed.

MATERIALS AND METHODS

General. Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were kindly donated by Novo Nordisk (Bagsvaerd, Denmark). Bio-Beads S-X3 were from Bio-Rad Laboratories (Munich, Germany), and the Bio-Beads size exclusion chromatography glass column ECO^{PLUS} was from Kronlab (Sinsheim, Germany). Sephadex LH-20 and the glass column were from Amersham Pharmacia Biotech (Freiburg, Germany). Size exclusion and Sephadex LH-20 chromatography instruments (L-6000 pump, L-7400 UV detector) were from Merck/Hitachi (Darmstadt, Germany). Phenyl-hexyl high-performance liquid chromatography (HPLC) columns were purchased from Phenomenex (Aschaffenburg, Germany), and the analytical RP-18 column (Prontosil 120-3-18ace-EPS) was from Bischhoff Chromatography (Leonberg, Germany). Phenyl-hexyl reversed-phase (RP) HPLC was carried out using the following instrumentation: L-6200 intelligent pump, T-6300 column thermostat, L-7400 preparative UV detector (Merck/Hitachi), or model 994 analytical photodiode array detector (Waters, Eschborn, Germany). HPLC-MS instrumentation was from Hewlett-Packard (Waldbronn, Germany), HP Series 1100: autosampler G1313, pump G 1312A, and mass spectrometer G 1946A. NMR experiments were performed on a Bruker DRX-500 (Rheinstetten, Germany) and a Varian Gemini-2000BB instrument (Palo Alto, CA).

Material. Maize bran (*Zea mays* L.) was kindly provided by Hammermühle Maismühle GmbH (Kirrweiler, Germany).

Preparation of Insoluble Maize Fiber. Acetone-extracted maize bran was used to prepare insoluble maize fiber by an enzymatic procedure (heat-stable α-amylase, protease, and amyloglucosidase) as described previously (*19*). From 380 g of maize bran, about 170 g of insoluble fiber was achieved.

Alkaline Hydrolysis and Extraction. Alkaline hydrolysis and extraction were carried out as described previously (*19*). In brief, insoluble maize fiber was saponified (2 M NaOH) under nitrogen and protected from light for 18 h. Following acidification of the mixture (pH < 2), liberated phenolic acids were extracted into diethyl ether. Ether extracts were extracted with $NaHCO_3$ solution (5%). The aqueous layers were carefully acidified (pH < 2), and phenolic acids were re-extracted into diethyl ether. Ether extracts were dried over Na_2SO_4 and evaporated to dryness. The residues were further dried under a stream of N_2 to give about 6 g of hydrolysate out of 380 g of maize bran (170 g of fiber).

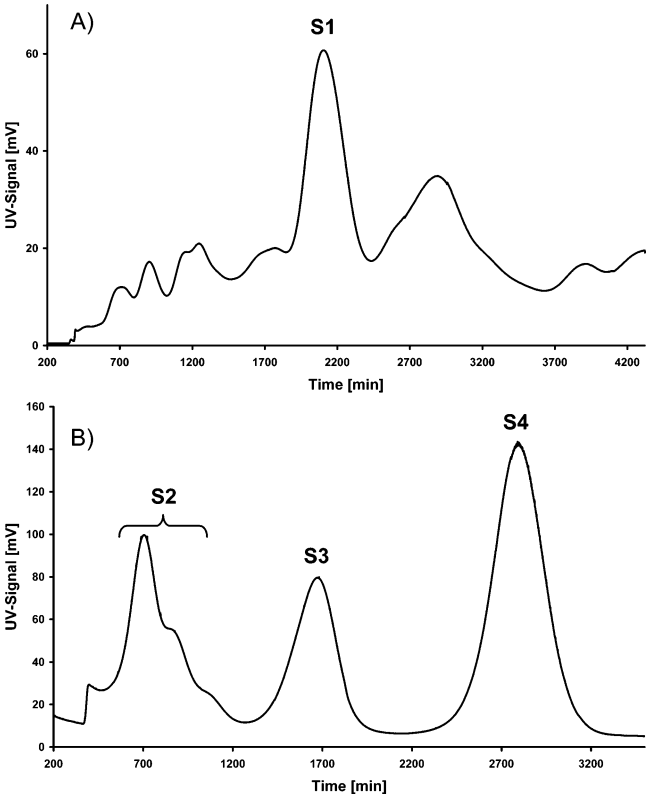

Figure 2. Sephadex LH-20 chromatogram of the Bio-Beads fraction B3 leading to a further separation of ferulate dehydrotrimers. (**A**) Elution with 0.5 mM aqueous trifluoroacetic acid/methanol (50/50, v/v) followed by (**B**) elution with 0.5 mM aqueous trifluoroacetic acid/methanol (40/60, v/v). Fraction S1 contained the known dehydrotrimers 8-*O*-4/8-5(noncyclic)-triferulic acid and 8-8(cyclic)/8-*O*-4-triferulic acid, S2 contained the known 8-*O*-4/8-*O*-4-dehydrotriferulic acid and compound **2**, S3 contained compound **1**, and fraction S4 contained pure 5-5/8-*O*-4-dehydrotriferulic acid.

Size Exclusion Chromatography. Preseparation of phenolic acids according to their oligomerization degree was carried out using size exclusion chromatography. A solvent resistant glass column with Teflon flow adaptors (1.5 cm × 100 cm) was filled with Bio-Beads S-X3 swollen in tetrahydrofuran that was also used as an eluent. About 300 mg of hydrolysate dissolved in 500 μL of tetrahydrofuran was applied using a six-way valve and a 700 μL sample loop. The flow rate was maintained at 0.25 mL/min for 375 min and subsequently enhanced to 0.5 mL/min to elute the monomers. Fractions were collected according to the chromatogram. Tetramers eluted between about 230 and 280 min, trimers between 281 and 311 min, dimers between 312 and 370 min, and monomers between 371 and 395 min (**Figure 1**). Fractions from about 20 runs were pooled and used for further separation procedures.

Sephadex LH-20 Chromatography. The Bio-Beads fraction expected to contain ferulate dehydrotrimers (B3, **Figure 1**) was further separated using two runs of Sephadex LH-20 chromatography under the conditions described (*23*) with some minor modifications. In brief, roughly half of the trimer fraction was dissolved in 14.5 mL of MeOH/H_2O (50/50, v/v) and the sample was applied to a 83 cm × 2 cm column preconditioned with 0.5 mM aqueous trifluoroacetic acid/MeOH (95/5, v/v). Because of the temporary precipitation of extracted material at the beginning of the separation, an interspace of about 1 cm between floating punch and gel bed was filled with solvent (*23*). Elution was carried out as follows: (i) elution with 0.5 mM trifluoroacetic acid/MeOH (95/5, v/v) for 72 h at a flow rate of 1.5 mL/min; (ii) elution with 0.5 mM trifluoroacetic acid/MeOH (50/50, v/v) for 72 h at a flow rate of 1.0 mL/min; (iii) elution with 0.5 mM trifluoroacetic acid/MeOH

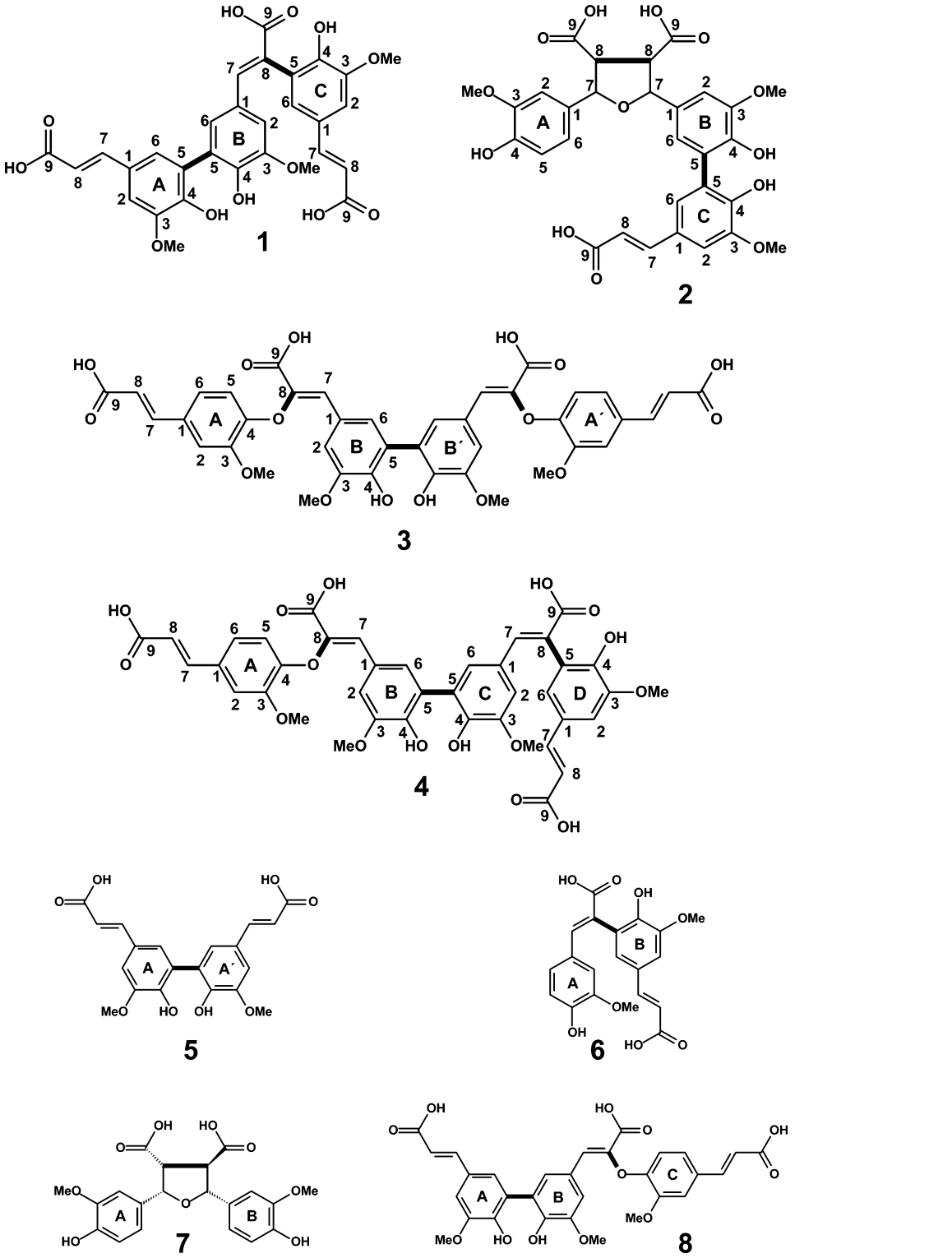

Figure 3. Chemical structures and numbering systems of the isolated and identified dehydrotriferulic acids **1** [8-5(noncyclic)/5-5-dehydrotriferulic acid] and **2** [8-8(tetrahydrofuran)/5-5-dehydrotriferulic acid], and the dehydrotetraferulic acids **3** (4-*O*-8/5-5/8-*O*-4-dehydrotetraferulic acid) and **4** [4-*O*-8/5-5/8-5(noncyclic)-dehydrotetraferulic acid]. The bond formed by the radical coupling step is bolded. NMR data of compounds **5**–**8** [**5**, 5-5-dehydrodiferulic acid; **6**, 8-5(noncyclic)-dehydrodiferulic acid; **7**, 8-8(tetrahydrofuran)-dehydrodiferulic acid; and **8**, 5-5/8-*O*-4-dehydrotriferulic acid] were used in **Tables 1**–**4** as reference data to ensure signal assignments of compounds **1**–**4**.

(40/60, v/v) for 65 h at a flow rate of 1.0 mL/min; and (iv) rinsing step with 0.5 mM trifluoroacetic acid/MeOH (10/90, v/v). Detection was carried out at 325 nm. Fractions were collected over 18 min periods.

Semipreparative RP-HPLC of Sephadex LH-20 Fractions (Ferulate Dehydrotrimers). Further separation of Sephadex LH-20 fractions S1, S2, and S3 (**Figure 2**) was achieved by means of semipreparative RP-HPLC using a 250 mm × 10 mm i.d., 5 μm, Luna phenyl-hexyl column. The column temperature was generally 35 °C, the flow rate was 2.5 mL/min, and detection was carried out at 325 nm. Sample loops of 100 or 200 μL were used, and 60 or 100 μL samples (dissolved in methanol), respectively, were injected. The eluents and gradient systems were as follows: eluent A, 1 mM aqueous trifluoroacetic acid; eluent B, MeOH/1 mM aqueous trifluoroacetic acid (90/10, v/v); and eluent C, acetonitrile/1 mM aqueous trifluoroacetic acid (90/10,

v/v). Gradient for separation of fraction S1: initially A 60%, B 25%, C 15%, held for 15 min, linear over 5 min to A 45%, B 35%, C 20%, held isocratically for 5 min, linear over 5 min to A 25%, B 35%, C 40%, held isocratically for 5 min, following rinsing and equilibration steps. The known ferulate dehydrotrimers 8-*O*-4/8-5(noncyclic)-triferulic acid and 8-8(cyclic)/8-*O*-4-triferulic acid eluted after 17.7 and 22.7 min, respectively. Gradient for separation of fraction S2: initially A 75%, B 10%, C 15%, held for 10 min, linear over 5 min to A 60%, B 25%, C 15%, held isocratically for 5 min, linear over 5 min to A 50%, B 25%, C 25%, held isocratically for 5 min, linear over 5 min to A 20%, B 25%, C 55%, held isocratically for 5 min, following an equilibration step. Compound **2** (**Figure 3**) and the known trimer 8-*O*-4/8-*O*-4-dehydrotriferulic acid eluted after 30.8 and 34.1 min, respectively. Gradient for separation of fraction S3: initially A 65%, B 20%, C 15%, held for 15 min, linear over 5 min to A 50%, B 35%, C 15%, held isocratically for 5 min, linear over 5 min to A 40%, B 35%, C 25%, linear over 5 min to A 10%, B 35%, C 55%, held isocratically for 5 min, following an equilibration step. Compound **1** (**Figure 3**) eluted after 24.7 min and was well-separated from the residual dimer 5-5-dehydrodiferulic acid (**5**, **Figure 3**).

Semipreparative RP-HPLC Separation of Ferulate Dehydrotetramers. Ferulate dehydrotetramers from the Bio-Beads fraction B4 (**Figure 1**) were separated by semipreparative RP-HPLC using the conditions as described for the ferulate dehydrotrimers. Deviating from the procedure described, fraction B4 was dissolved in acetone/water (5/3, v/v) for injection. The gradient was used as follows: eluent A, 1 mM aqueous trifluoroacetic acid; eluent B, MeOH/1 mM aqueous trifluoroacetic acid 90/10 (v/v); and eluent C, acetonitrile/1 mM aqueous trifluoroacetic acid 90/10 (v/v); initially A 70%, B 25%, C 5%, linear over 90 min to A 30%, B 65%, C 5%, following rinsing and equilibration steps. Compound **4** eluted after 53.2 min, and compound **3** (**Figure 3**) eluted after 67.4 min.

HPLC-MS. Molecular mass determination was achieved by HPLC-MS using atmospheric pressure-electrospray ionization (AP-ESI) in the positive and negative modes [fragmentor voltage: 90 V (negative mode) and 75 V (positive mode); scan range, *m*/*z* 100−1000]. Fast elution of the isolated trimers and tetramers following semipreparative RP-HPLC on a 150 mm × 3 mm i.d., 3 μm, Prontosil RP18 column was carried out by the following HPLC conditions: eluent A, 0.1% aqueous formic acid; eluent B, acetonitrile. Initially A 65%, B 35%, linear over 10 min to A 55%, B 35%, linear over 5 min to A 10%, B 90%, following an equilibration step. The injection volume was 20 μL, and the flow rate was maintained at 0.6 mL/min.

Positive-ion mode AP-ESI MS, peaks > 10% of base peak, *m*/*z* (% of base peak). Compound **1**: 617 $[M + K]^+$ (19), 601 $[M + Na]^+$ (100), 525 (76), 507 (18). Compound **2**: 635 $[M + K]^+$ (51), 619 $[M + Na]^+$ (56), 561 (27), 543 (30), 533 (30), 525 (15), 515 (100), 497 (52), 463 (30), 445 (51), 419 (24), 393 (18). Compound **3**: 809 $[M + K]^+$ (6), 793 $[M + Na]^+$ (12), 767 (28), 735 (26), 717 (22), 693 (58), 424 (14), 402 (76), 384 (100). Compound **4**: 809 $[M + K]^+$ (19), 793 $[M + Na]^+$ (26), 735 (14), 717 (100).

Negative-ion mode AP-ESI MS, peaks > 5% of base peak, *m*/*z* (% of base peak). Compound **1**: 577 $[M - H]^-$ (79), 533 (100), 489 (11). Compound **2**: 595 $[M - H]^-$ (100), 551 (15). Compound **3**: 769 $[M - H]^-$ (100). Compound **4**: 769 $[M - H]^-$ (100), 725 (23).

Structural Identification by NMR. Structural identification was performed using the usual array of one- and two-dimensional (1D and 2D) NMR experiments [^{1}H, ^{13}C, H,H-correlated spectroscopy (COSY), heteronuclear mulitple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC)]. One-dimensional ^{13}C spectra were acquired using the attached proton test (ATP) experiment. Because of low sample amounts (around or less than 1 mg), ^{13}C spectra were generally weak, especially signals for quaternary carbons. Although ^{13}C data were acquired for 50−60 h, some spectra were too weak to extract ^{13}C data from the 1D experiments. In these cases, ^{13}C data were taken from the 2D experiments HMQC and HMBC. Samples were dissolved in 0.7 mL of acetone-d_6. Chemical shifts (δ) were referenced to the central solvent signals (δ_H 2.04 ppm; δ_C 29.8 ppm). *J* values are given in Hz. NMR assignments follow the numbering shown in **Figure 3**.

RESULTS AND DISCUSSION

Separation Procedure. Before the present study, five ferulic acid dehydrotrimers [5-5/8-*O*-4-, 8-*O*-4/8-*O*-4-, 8-8(cyclic)/8-*O*-4-, 8-*O*-4/8-5(noncyclic)-, and 5-5/8-*O*-4(H_2O)-dehydrotriferulic acids] had been isolated from maize bran (*19*−*22*). For this, we used a sequence of Sephadex LH-20 chromatography and RP-HPLC, originally developed for the isolation of dehydrodiferulic acids, and 5-5/8-*O*-4-dehydrotriferulic acid as standard compounds (*23*). Sephadex LH-20 chromatography was suitable for separating phenolic monomers from dimers and trimers, but a separation of dimers and trimers based on their molecular mass has not been possible. For a more distinct separation depending on the molecular size, we used size exclusion chromatography on Bio-Beads S-X3, a method developed for the separation of oligomers resulting from model reactions using ethyl ferulate as substrate (unpublished results). As shown in **Figure 1**, phenolic monomers (B1) separated well from dimers (B2) and trimers (B3). Phenolic tetramers (B4) partially coeluted with the trimer fraction. Although phenolic monomers separated well from the oligomer fractions, small amounts of ferulic acid were found in all fractions. To date, we can only speculate whether ferulic acid is loosely attached to phenolic oligomers, thus being dragged into the oligomer fractions or to the stationary phase, thus leading to a small but continuous elution of monomeric ferulic acid. Furthermore, it is possible that real oligomers are decomposed setting monomeric ferulic acid free. Application of semipreparative RP-HPLC on the trimer fraction B3 using a phenyl-hexyl stationary phase led to complex chromatograms, and it was not possible to obtain pure compounds. Therefore, fraction B3 was further separated using the already mentioned Sephadex LH-20 procedure (**Figure 2**). Fraction S4 was pure 5-5/8-*O*-4-dehydrotriferulic acid, and from RP-HPLC separation of S1, the already known trimers 8-*O*-4/8-5(noncyclic)-triferulic acid and 8-8(cyclic)/8-*O*-4-triferulic acid were obtained. Fraction S3 was further separated by RP-HPLC to give compound **1** (**Figure 3**) and small amounts of 5-5-dehydrodiferulic acid. RP-HPLC of fraction S2 gave the known 8-*O*-4/8-*O*-4-triferulic acid and compound **2**. From the tetramer fraction B4, compounds **3** and **4** were obtained by RP-HPLC without further separation steps. Compound **3** was quite pure whereas compound **4** still contained some impurities but was sufficiently pure to be identified by HPLC-MS and NMR.

Structural Identification of Compound 1 {8-5(Noncyclic)/5-5-Dehydrotriferulic Acid; 3-[5′-(2-Carboxy-vinyl)-6,2′-dihydroxy-5,3′-dimethoxy-biphenyl-3-yl]-2-[5-(2-carboxy-vinyl)-2-hydroxy-3-methoxy-phenyl]acrylic Acid}. The UV spectrum of compound **1** shows characteristics of ferulic acid-related structures (**Figure 4A**). The molecular mass determined by HPLC-ESI-MS indicated a ferulic acid dehydrotrimer. Negative ion MS gave a high mass of *m*/*z* 577 $[M - H]^-$ suggesting a molecular mass of 578. Positive ion MS showed a base peak of *m*/*z* 601, deriving from the sodium adduct ion $[M + Na]^+$ along with a peak of *m*/*z* 617 $[M + K]^+$ (19% of base peak), both suggesting a molecular mass of 578 and indicating a triferulic acid structure.

Using 1D and 2D NMR (HMQC, HMBC, and H,H COSY) experiments, compound **1** was unambiguously identified as 8-5(noncyclic)/5-5-dehydrotriferulic acid. The proton spectrum showed the signals of 20 protons attached to carbons (from HMQC). Nine protons belong to three methoxyl groups. Some of the quaternary carbons in the 1D carbon spectrum, e.g., carbons in 4-positions, were too weak to be deduced. These carbon shifts were revealed and taken from the 2D HMBC spectra. The carbon spectrum revealed three carboxyl groups

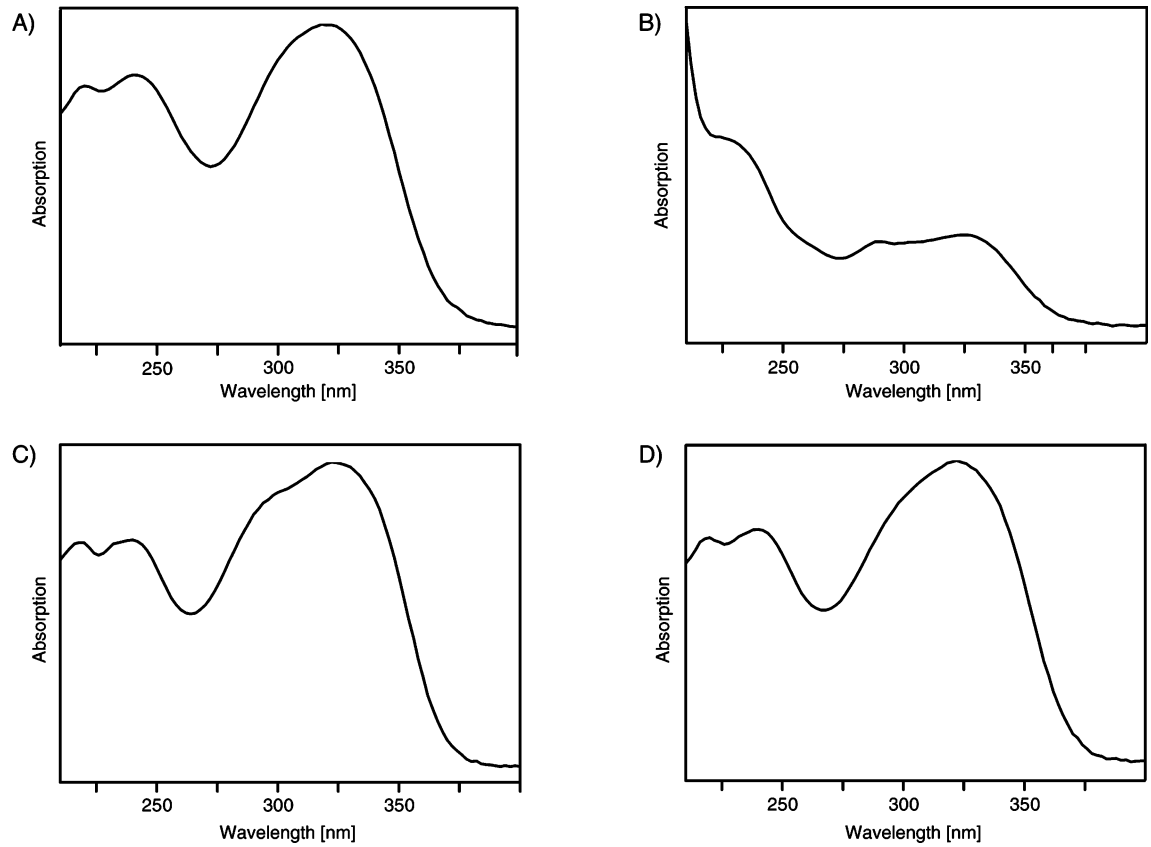

Figure 4. UV spectra of (**A**) compound **1** [8-5(noncyclic)/5-5-dehydrotriferulic acid], (**B**) compound **2** [8-8(tetrahydrofuran)/5-5-dehydrotriferulic acid], (**C**) compound **3** (4-*O*-8/5-5/8-*O*-4-dehydrotetraferulic acid), and (**D**) compound **4** [4-*O*-8/5-5/8-5(noncyclic)-dehydrotetraferulic acid].

(chemical shifts around 168 ppm) thus supporting the MS data suggesting a dehydrotriferulic acid. Four 15.9 Hz doublets in the proton spectrum indicate two unsubstituted *trans*-cinnamic acid side chains, and a singlet at 7.86 ppm characteristic of unsaturated 7-protons suggests one coupling via the 8-position. All remaining aromatic proton signals are doublets showing coupling constants of 1.8 or 1.9 Hz. The absence of 8 Hz doublets, resulting from H5−H6 coupling in guaiacyl units, points out that all ferulate moieties are coupled via their 5-position. As a consequence, the ferulate moieties have to be coupled via one 5-5-linkage and via one 8-5(noncyclic)-linkage. From the HMBC experiment, the propenyl side chains were associated with the corresponding aromatic rings. All correlation signals in the HMBC fully align with the structure shown in **Figure 3**. However, it is difficult to assign ring A and ring C, since both have very similar spectroscopic properties (**Table 1**). Problems are the signal diversity between 125 and 128.5 ppm and one missing signal in the 1D carbon spectrum in this region. To make sure that this signal was not just missing due to the phase sensitivity of the ATP experiment (cancellation of a positive (weak) quaternary signal by a negative signal from a tertiary carbon), the experiment suppressing all signals other than those from quaternary carbons was run. However, no additional quaternary signals arose. From this experiment and the HMBC data, we suppose that the carbon signals BC5 and CC5 are superimposed. From detailed analysis of the HMBC spectrum and comparison with model compounds (**Table 1**), we assigned chemical shifts to the rings A and C as shown in **Table 1**. The only surprising chemical shift is the proton shift of A6 that is significantly lower than in the reference model compound; molecular modeling readily indicates that ring C is shielding this proton.

Structural Identification of Compound 2 {8-8(Tetrahydrofuran)/5-5-Dehydrotriferulic Acid; 2-[5′-(2-Carboxyvinyl)-6,2′-dihydroxy-5,3′-dimethoxy-biphenyl-3-yl]-5-(4-hydroxy-3-methoxy-phenyl)tetrahydrofuran-3,4-dicarboxylic Acid}. The UV spectrum of compound **2** shows two broad maxima around 288 and 324 nm, the second one being characteristic for ferulic acid-related structures (**Figure 4B**). However, the spectrum differs considerably from other ferulate dimers, trimers, and tetramers, e.g., the other spectra in **Figure 4**. The relatively low absorption around 320−325 nm hints at modifications in the propenyl side chains of the ferulates. The molecular mass determined by HPLC-ESI-MS exceeds the mass of a ferulic acid dehydrotrimer by 18. Negative ion MS gave a high mass of *m*/*z* 595 $[M - H]^-$ (base peak) suggesting a molecular mass of 596. Positive ion MS showed the potassium adduct ion of *m*/*z* 635 $[M + K]^+$ (51% of base peak) and the sodium adduct ion of *m*/*z* 619 $[M + Na]^+$ (56% of base peak), also suggesting a molecular mass of 596, leading to the contention that this compound is a water addition product. The structure of compound **2** shown in **Figure 3** was unambiguously identified by using 1D and 2D NMR. Twenty-two proton signals were observed in the 1D proton spectrum; three singlets were assigned to nine protons from three methoxyl groups, and two 15.9 Hz doublets indicate one unsubstituted propenyl side chain of a *trans*-ferulate moiety. Seven signals from aromatic protons were identified, and their coupling constants indicate two guaiacyl units coupled via their 5-positions and one guaiacyl unit with protons in 2-, 5-, and 6-positions (i.e., not coupled via its 5-position). In addition, characteristic proton doublets were found at 5.36 and 5.40 ppm coupling to proton signals at 3.58 and 3.64 ppm, respectively. The latter signals also couple with each other. Two carbon signals at 172.3 ppm and one

Table 1. NMR Data for Compound **1**, 8-5(Noncyclic)/5-5-dehydrotriferulic Acid, δ in ppm and J in Hz[a]

	^{1}H		^{13}C	
trimer unit	in acetone-d_6	ref in acetone-d_6	in acetone-d_6	ref in acetone-d_6
A1			126.8!!!	126.6[b]
A2	7.32 (1H, d, 1.9)	7.35 (1H, d, 2.0)[b]	109.7	110.0[b]
A3			148.9!!	148.9[b]
A4			146.9[d]	147.4[b]
A5			125.7	125.6[b]
A6	6.86 (1H, d, 1.9)	7.21 (1H, d, 2.0)[b]	126.2	126.1[b]
A7	7.60 (1H, d, 15.9)	7.64 (1H, d, 15.9)[b]	146.0	145.9[b]
A8	6.39 (1H, d, 15.9)	6.42 (1H, d, 15.9)[b]	116.1	116.3[b]
A9			168.1!!!!	168.4[b]
A3-OMe	3.94 (3H, s)!	3.97 (3H, s)[b]	56.5	56.5[b]
B1			127.0	127.6[e]
B2	6.82 (1H, d, 1.8)		112.4	113.3[e]
B3			148.0	147.9[e]
B4			146.2[d]	149.0[e]
B5			125.2	115.6[e]
B6	6.91 (1H, d, 1.8)		128.3	126.4[e]
B7	7.86 (1H, s)		141.7	141.8[e]
B8			126.7!!!	126.3[e]
B9			168.5	169.2[e]
B3-OMe	3.57 (3H, s)		55.8	55.5[e]
C1			127.4	127.3[c]
C2	7.42 (1H, d, 1.9)	7.37 (1H, d, 1.9)[c]	110.1	110.3[c]
C3			149.1!!	149.1[c]
C4			148.0[d]	148.0[c]
C5			125.2	125.1[c]
C6	7.06 (1H, d, 1.9)	7.03 (1H, d, 1.9)[c]	125.9	125.6[c]
C7	7.61 (1H, d, 15.9)	7.60 (1H, d, 15.9)[c]	145.6	145.8[c]
C8	6.43 (1H, d, 15.9)	6.38 (1H, d, 15.9)[c]	116.4	116.2[c]
C9			168.0!!!!	168.6[c]
C3-OMe	3.95 (3H, s)!	3.95[c]	56.5	56.6[c]

[a] !, !!, !!!, and !!!!: assignments may be interchanged. Blank cells, no adequate reference data available. Abbreviations used: s, singlet; d, doublet. [b] Reference data from 5-5-dehydrodiferulic acid (compound **5**, **Figure 3**) (*10*). [c] Reference data taken from 8-5(noncyclic)-dehydrodiferulic acid (ferulate ring B, compound **6**, **Figure 3**) (*10*). [d] Because 4-carbons are too weak in the 1D experiment, values are taken from the 2D HMBC data. [e] Reference data taken from 8-5(noncyclic)-dehydrodiferulic acid (ferulate ring A, compound **6**, **Figure 3**) (*10*). Note that compound **1** has an additional 5-linkage in ring B.

carbon signal at 167.5 ppm (**Table 2**) taken from the HMBC spectrum indicated carboxylic groups, one of them (signal at 167.5 ppm) being part of an unsubstituted propenyl side chain, whereas the other ones couple to the characteristic protons with signals around 5.4 and 3.6 ppm, leading to the assumption that these protons are in 7- and 8-positions of modified side chains. HMBC correlation peaks showing coupling of 7-protons with signals at 5.36 and 5.40 ppm to A6/A2 and B6/B2 carbons, respectively, confirmed this assignment. These findings in conjunction with the carbon shifts (**Table 2**) and the mass indicating the incorporation of an additional water showed the coupling of ferulates A and B via an 8-8/7-*O*-7-unit. This type of unit was recently also found to be an authentic product of 8-8-coupling of ferulates (*24*) (**Figure 3**, compound **7**) which has been designated as 8-8(tetrahydrofuran)-coupling. Coupling of ferulates B and C is via a 5-5-linkage. All correlation peaks in the HMBC and all spectroscopic data shown in **Table 2** are fully consistent with our structural identification of compound **2** as 8-8(tetrahydrofuran)/5-5-dehydrotriferulic acid.

Structural Identification of Compound 3 {**4-*O*-8/5-5/8-*O*-4-Dehydrotetraferulic Acid; 3-(5′-{2-Carboxy-2-[4-(2-carboxy-vinyl)-2-methoxy-phenoxy]vinyl}-6,2′-dihydroxy-5,3′-dimethoxy-biphenyl-3-yl)-2-[4-(2-carboxy-vinyl)-2-methoxy-phenoxy]acrylic Acid**}**.** The UV spectrum of compound **3** is typical for a ferulate derivative (**Figure 4C**). The relatively broad but distinct shoulder between 290 and 315 nm is characteristic of 8-*O*-4-coupled ferulates (*19*, *25*). The mass spectrum indicated a dehydrotetraferulate structure. Negative ion MS produced an $[M - H]^-$ of *m*/*z* 769 whereas positive ion MS gave high masses of 809 $[M + K]^+$ and 793 $[M + Na]^+$ suggesting a molecular mass of 770. The NMR spectra showed surprisingly few signals immediately suggesting a tetrameric structure containing a center of symmetry. Signals integrated to 28 protons, 12 of them assigned to two sets of two methoxyls. Two 16.0 Hz doublets at 7.59 and 6.43 ppm indicated one pair of unsubstituted *trans*-cinnamate side chains, whereas the singlet at 7.44 ppm, in conjunction with its corresponding carbon shift of 128.7 ppm, is a prime indicator that one pair of units is 8-*O*-4-linked. One doublet-of-doublets at 7.13 ppm with coupling constants of 8.3 and 1.9 Hz indicates guaiacyl units with protons in 2-, 5-, and 6-positions, whereas the two 1.9 Hz doublets at 7.55 and 7.20 ppm indicate that the second ferulate pair is 5-linked. The HMBC spectrum revealed that the unsubstituted cinnamate side chains are bound to the guaiacyl units containing protons in 2-, 5-, and 6-positions, whereas the 8-*O*-4-linked side chain is linked to guaiacyl units linked via their 5-positions. Full analysis of the NMR spectra provided the assignments shown in **Table 3**, consistent with compound **3**, in which there is 5-5-coupling of the interior unit B with unit B′, as shown in **Figure 3**. Units A′ and B′ represent the symmetry counterparts to units A and B. Units B and C of 5-5/8-*O*-4-dehydrotriferulic acid (compound **8**, **Figure 3**) are good models for the units A and B of compound **3**. Because we formerly provided NMR data for compound **8** only in acetone-d_6/D_2O 3/1 and in methanol-d_4 (*21*), we also measured compound **8** in acetone-d_6 here. NMR data for units B and C are given as reference data in **Table 3**; the chemical shifts of compound **3** are in good agreement with the reference data from compound **8**. As in the case of compound **1**, the only chemical shift that is not well-modeled is the proton shift of B6, showing that the aromatic ring in unit A′ shields proton-6 of unit B.

Structural Identification of Compound 4 {**4-*O*-8/5-5/8-5-(Noncyclic)-dehydrotetraferulic Acid; 3-(5′-{2-Carboxy-2-[5-(2-carboxy-vinyl)-2-hydroxy-3-methoxy-phenyl]vinyl}-6,2′-dihydroxy-5,3′-dimethoxy-biphenyl-3-yl)-2-[4-(2-carboxy-vinyl)-2-methoxy-phenoxy]acrylic Acid**}**.** Unlike compounds **1**−**3**, which were isolated in >90% purity (as estimated by ^{1}H NMR), compound **4** is estimated to be around 75% pure. Although the impurities did not interfere in the structural elucidation of compound **4**, we here describe the identification of its structure as tentative. The UV spectrum of compound **4** consistent with a ferulic acid derivative is shown in **Figure 4D**. The molecular mass of compound **4** was determined by HPLC-MS to be 770, with a high mass of *m*/*z* 769 $[M - H]^-$ in the negative ion mode and high masses of *m*/*z* 809 $[M + K]^+$ and *m*/*z* 793 $[M + Na]^+$ in the positive ion mode. Proton NMR in concert with carbon shifts deduced from the HMQC spectrum revealed four methoxyl groups. Four doublets with coupling constants of 15.9 Hz show two unsubstituted *trans*-cinnamate side chains. Two singlets at 7.42 (in conjunction with the carbon shift at 128.6 ppm) and 7.83 ppm (in conjunction with the carbon shift at 141.9 ppm) are indicators for 8-coupling (8-*O*-4- and 8-5-coupling, respectively) of these ferulate moieties. One doublet-of-doublets at 7.14 ppm in conjunction with the COSY-correlated 8.3 Hz doublet at 6.84 ppm and the 2.0 Hz doublet at 7.44 ppm points out that only one guaiacyl unit has protons in 2-, 5-, and 6-positions thus being linked via its phenolic group. The HMBC spectrum showed that this guaiacyl unit (unit A in **Figure 3**, compound **4**) is linked to an unsubstituted side chain.

Table 2. NMR Data for Compound **2**, 8-8(Tetrahydrofuran)/5-5-dehydrotriferulic Acid, δ in ppm and J in Hz[a]

	^{1}H		^{13}C	
trimer unit	in acetone-d_6	ref in acetone-d_6	in acetone-d_6	ref in acetone-d_6
A1			132.9[d]	130.5[b]
A2	7.13 (1H, d, 1.7)	7.09 (1H, d, 2.0)[b]	110.1[e]	111.5[b]
A3			147.7[d]	147.8[b]
A4			146.7[d]	147.1[b]
A5	6.81 (1H, d, 8.1)	6.77 (1H, d, 8.2)[b]	115.0[e]	115.2[b]
A6	6.94 (1H, dd, 8.1, 1.7)	6.94 (1H, dd, 8.2, 2.0)[b]	119.6[e]	120.8[b]
A7	5.36 (1H, d, 8.4)	5.31 (1H, d, 8.6)[b]	83.6[e]	82.9[b]
A8	3.58 (1H, dd, 9.7, 8.4)	3.80 (1H, d, 8.6, 6.6)[b]	57.5[e]	55.4[b]
A9			172.3[d]	172.9[b]
A3-OMe	3.85 (3H, s)	3.81/3.87 (3H, s)	55.8[d]	56.1/56.2[b]
B1			(132.5)[d]	130.5[b]
B2	7.16 (1H, d, 1.8)		109.0[e]	111.5[b]
B3			148.1[d]	147.8[b]
B4			144.0[d]	147.1[b]
B5			124.5[d]	115.2[b]
B6	7.04 (1H, d, 1.8)		121.7[e]	120.8[b]
B7	5.40 (1H, d, 7.9)		83.7[e]	82.9[b]
B8	3.64 (1H, dd, 9.7, 7.9)		57.2[e]	55.4[b]
B9			172.3[d]	172.9[b]
B3-OMe	3.91 (3H, s)		56.0[e]	56.1/56.2[b]
C1			126.2[d]	126.6[c]
C2	7.34 (1H, d, 1.9)	7.35 (1H, d, 1.9)[c]	109.2[e]	110.0[c]
C3			148.5[d]	148.9[c]
C4			146.4[d]	147.4[c]
C5			125.8[d]	125.6[c]
C6	7.18 (1H, d, 1.9)	7.21 (1H, d, 1.9)[c]	125.7[e]	126.1[c]
C7	7.63 (1H, d, 15.9)	7.64 (1H, d, 15.9)[c]	145.4[e]	145.9[c]
C8	6.41 (1H, d, 15.9)	6.42 (1H, d, 15.9)[c]	115.6[e]	116.3[c]
C9			167.5[d]	168.4[c]
C3-OMe	3.96 (3H, s)	3.97[c]	56.0[e]	56.5[c]

[a] Blank cells, no adequate reference data available. Abbreviations used: s, singlet; d, doublet; and dd, doublet of doublets. [b] Reference data taken from 8-8-(tetrahydrofuran)-dehydrodiferulic acid (ferulate ring A, compound **7**, **Figure 3**) (*24*). Note that this model does not reflect additional 5-linkage of ring B of compound **2**. [c] Reference data taken from 5-5-dehydrodiferulic acid (compound **5**, **Figure 3**) (*10*). [d] Data taken from 2D HMBC data. [e] Data taken from 2D HMQC data.

Table 3. NMR Data for Compound **3**, 4-*O*-8/5-5/8-*O*-4-Dehydrotetraferulic Acid, δ in ppm and J in Hz[a]

	^{1}H		^{13}C	
tetramer unit	in acetone-d_6	ref in acetone-d_6	in acetone-d_6	ref in acetone-d_6[b]
A1			130.2[d]	130.1[b]
A2	7.43 (1H, d, 1.9)	7.40 (1H, d, 1.7)[b]	112.2	112.4[b]
A3			150.3[d]	150.2[b]
A4			149.0[d]	148.9[b]
A5	6.84 (1H, d, 8.3)	6.83 (1H, d, 8.4)[b]	114.3	114.4[b]
A6	7.13 (1H, dd, 8.3, 1.9)	7.12 (1H, dd, 8.4, 1.7)[b]	122.9	122.9[b]
A7	7.59 (1H, d, 16.0)	7.58 (1H, d, 15.9)[b]	145.3	145.2[b]
A8	6.43 (1H, d, 16.0)	6.41 (1H, d, 15.9)[b]	117.5	117.6[b]
A9			168.0[d]	168.3[b]
A3-OMe	3.94 (3H, s)	3.92 (3H, s)[b]	56.3	56.4[b]
B1			(124.5)[e]	124.6[c]
B2	7.55 (1H, d, 1.9)	7.52 (1H, d, 1.9)[c]	112.3	112.6[c]
B3			148.6[d]	148.6[c]
B4			147.1[d]	147.0[c]
B5			125.7[d]	125.8[c]
B6	7.20 (1H, d, 1.9)	7.32 (1H, d, 1.9)[c]	128.4	128.2[c]
B7	7.44 (1H, s)	7.45 (1H, s)[c]	128.7	128.5[c]
B8			138.6[d]	138.7[c]
B9			164.6[d]	164.8[c]
B3-OMe	3.76 (3H, s)	3.77 (3H, s)[c]	56.1	56.2[c]

[a] Abbreviations used: s, singlet; d, doublet; and dd, doublet of doublets. [b] Reference data from 5-5/8-*O*-4-dehydrotriferulic acid (*21*, *22*) (ferulate unit C, compound **8**, **Figure 3**) in acetone-d_6 (see text). [c] Reference data from 5-5/8-*O*-4-dehydrotriferulic acid (*21*, *22*) (ferulate unit B, compound **8**, **Figure 3**) in acetone-d_6 (see text). [d] Because quaternary carbon signals were generally weak in 1D ^{13}C experiment, values are taken from the 2D HMBC data. [e] Weak signal in the ^{13}C spectrum, but no correlation peaks in the HMBC spectrum.

Six more doublets in the proton spectrum with coupling constants of 1.9 Hz indicate that three ferulate moieties are coupled via their 5-position. From these data, we suggested compound **4** to be 4-*O*-8/5-5/8-5-(noncyclic)-dehydrotetraferulic acid (**Figure 3**). Full analysis of the 2D experiments led to the signal assignment given in **Table 4**, supporting our structural

Table 4. NMR Data for Compound **4**, 4-*O*-8/5-5/8-5(Noncyclic)-dehydrotetraferulic Acid, δ in ppm and *J* in Hz[a]

	^{1}H		^{13}C	
tetramer unit	in acetone-d_6	ref in acetone-d_6	in acetone-d_6	ref in acetone-d_6
A1			130.1[f]	130.1[b]
A2	7.44 (1H, d, 2.0)	7.40 (1H, d, 1.7)[b]	112.3[f]	112.4[b]
A3			150.1[f]	150.2[b]
A4			148.9[f]	148.9[b]
A5	6.84 (1H, d, 8.3)	6.83 (1H, d, 8.4)[b]	114.1[g]	114.4[b]
A6	7.14 (1H, dd, 8.3, 2.0)	7.12 (1H, dd, 8.4, 1.7)[b]	122.9[f]	122.9[b]
A7	7.59 (1H, d, 15.9)	7.58 (1H, d, 15.9)[b]	145.3[f]	145.2[b]
A8	6.43 (1H, d, 15.9)	6.41 (1H, d, 15.9)[b]	117.7[f]	117.6[b]
A9			168.0[f]	168.3[b]
A3-OMe	3.96 (3H, s)	3.92 (3H, s)[b]	55.9[g]	56.4[b]
B1			?	124.6[c]
B2	7.54 (1H, d, 1.9)	7.52 (1H, d, 1.9)[c]	112.1[f]	112.6[c]
B3			148.5[f]	148.6[c]
B4			146.9[f]	147.0[c]
B5			125.7[f]	125.8[c]
B6	6.95 (1H, d, 1.9)	7.32 (1H, d, 1.9)[c]	128.6[f]	128.2[c]
B7	7.42 (1H, s)	7.45 (1H, s)[c]	128.6[f]	128.5[c]
B8			138.5[f]	138.7[c]
B9			164.7[f]	164.8[c]
B3–OMe	3.73 (3H, s)	3.77 (3H, s)[c]	55.9[g]	56.2[c]
C1			?	127.0[d]
C2	6.78 (1H, d, 1.9)	6.82 (1H, d, 1.8)[d]	112.3[f]	112.4[d]
C3			148.1[f]	148.0[d]
C4			146.3[f]	146.2[d]
C5			125.2[f]	125.2[d]
C6	6.82 (1H, d, 1.9)	6.91 (1H, d, 1.8)[d]	128.3[f]	128.3[d]
C7	7.83 (1H, s)	7.86 (1H, s)[d]	141.9[f]	141.7[d]
C8			126.8[f]	126.7[d]
C9			168.6[f]	168.5[d]
C3–OMe	3.54 (3H, s)	3.57 (3H, s)[d]	55.4[g]	55.8[d]
D1			127.4[f]	127.4[e]
D2	7.45 (1H, d, 1.9)	7.42 (1H, d, 1.9)[e]	110.2[f]	110.1[e]
D3			149.3[f]	149.1[e]
D4			148.1[f]	148.0[e]
D5			125.2[f]	125.2[e]
D6	7.07 (1H, d, 1.9)	7.06 (1H, dd, 8.3, 1.9)[e]	126.0[f]	125.9[e]
D7	7.62 (1H, d, 15.9)	7.61 (1H, d, 15.9)[e]	145.7[f]	145.6[e]
D8	6.44 (1H, d, 15.9)	6.43 (1H, d, 15.9)[e]	116.2[f]	116.4[e]
D9			168.0[f]	168.0[e]
D3-OMe	3.98 (3H, s)!	3.95 (3H, s)[e]	56.1[g]	56.5[e]

[a] Abbreviations used: s, singlet; d, doublet; and dd, doublet of doublets. [b] Reference data from 5-5/8-*O*-4-dehydrotriferulic acid (*21*, *22*) (ferulate unit C, compound **8**, **Figure 3**) in acetone-d_6 (see text). [c] Reference data from 5-5/8-*O*-4-dehydrotriferulic acid (*21*, *22*) (ferulate unit B, compound **8**, **Figure 3**) in acetone-d_6 (see text). [d] Reference data from compound **1** [8-5(noncyclic)/5-5-dehydrotriferulic acid] (**Table 1**) (ferulate unit B) in acetone-d_6. [e] Reference data from compound **1** [8-5(noncyclic)/5-5-dehydrotriferulic acid] (**Table 1**) (ferulate unit C) in acetone-d_6. [f] Data taken from 2D HMBC data. [g] Data taken from 2D HMQC data.

proposal for compound **4**. Just as for compound **3**, units C and B from compound **8** are good models for the A and B units of compound **4**, and units B and C of compound **1** are good models for the units C and D of compound **4**. **Table 4** shows good agreement between the reference data of the models used and the data for compound **4**, again with the exception of the shielded proton-6 signals of the interior units B and C.

Possible Formation Pathways for Compounds 1−4 and Implications for Cell Wall Cross-Linking. The five dehydrotriferulates isolated previously (*19*−*22*) contain at least one 8-*O*-4-linkage, whereas only two of them contain a 5-5-linkage [5-5/8-*O*-4-dehydrotriferulic acid, compound **8**; 5-5/8-*O*-4-(H_2O)-dehydrotriferulic acid]. This was contrary to our first working hypothesis that all trimers should contain a 5-5-linkage, based on Hatfield's observation (*26*) that only 5-5-coupled dimers could be formed intramolecularly. Trimers involving 5-5-diferulate units were therefore envisioned to result from coupling between the diferulate on one arabinoxylan chain and a ferulate monomer on another; this would result in cross-linking only two polysaccharide chains, avoiding the need to contemplate the steric implications involved in coupling three ferulates independently bound to unwieldy polysaccharide chains. Hatfield's model using a xylan backbone of 16 xylose units reflected arabinoxylans that are described as rather rigid or at most semiflexible (*27*). However, recently, some evidence was supplied that xylans are able to be coiled or folded (*28*), invoking the possibility that linkages other than the 5-5-linkage might possibly form intramolecularly. Studies from Obel et al. (*29*) indicated that intracellular formation of dimers is limited to 8-5-coupling, suggesting that intracellular 8-5-coupling may be favored intramolecularly. When non-5-5-containing TFAs were discovered, we also realized that a "back-crossing" mechanism might still allow trimers to form between ferulates on just two chains, i.e., that cross-linking of three polysaccharide chains was still not mandated (*1*). In fact, all of the first trimers isolated and identified unexpectedly contained 8-*O*-4-linkages, reminiscent of the predominance of such linkages between monolignols in lignin polymers. Here, we isolated the first trimers that do not contain an 8-*O*-4-linkage. Compound **1** probably does not exist in its isolated form in the plant. In analogy to 8-5-dimers, it has to be assumed that compound **1** is formed from an 8-5(cyclic)/5-5-dehydrotriferulate during the saponification pro-

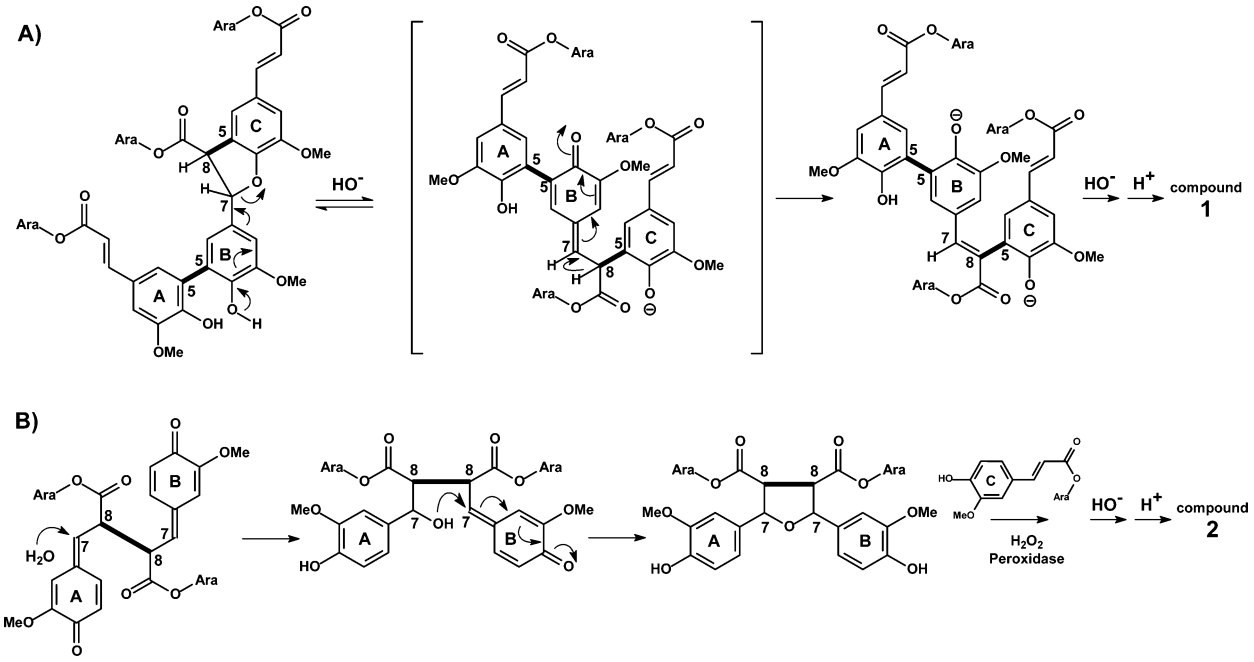

Figure 5. (**A**) Possible mechanism of the formation of compound **1** [8-5(noncyclic)/5-5-dehydrotriferulic acid] from its supposed precursor in the plant 8-5(cyclic)/5-5-dehydrotriferulate during the saponification process. (**B**) Formation of an 8-8/7-*O*-7-coupled dimeric moiety, a structural unit in compound **2** [8-8(tetrahydrofuran)/5-5-dehydrotriferulic acid]. Water is added to one quinone methide moiety. Subsequently, the newly formed 7-OH internally attacks on carbon 7 of the other quinone methide moiety. Note that the reaction sequence leading to the trimer is not definite; preformation of a 5-5-coupled dimer is also possible. Ara, arabinose from an arabinoxylan chain.

cess (**Figure 5A**). In fact, the parent phenylcoumaran ester in **Figure 5A** was a major compound resulting from radical coupling of ethyl ferulate with the diethyl ester of 5-5-dehydrodiferulic acid (unpublished results). Unlike with the formerly isolated trimers 8-8(cyclic)/8-*O*-4-dehydrotriferulic acid and 8-5(noncyclic)/8-*O*-4-dehydrotriferulic acid, it is not possible to give a formation sequence for compound **1** or its precursor. Either the 5-5-dimer or the 8-5-dimer can be formed in a first step without impeding the subsequent formation of the 8-5- or 5-5-linkage, respectively. The same is true for compound **2**. Either the 8-8/7-*O*-7- or the 5-5-linkage can be formed in the first step without impeding the formation of the other linkage in a second step. Trimer **2** contains a dimeric moiety that involves water addition to one quinone methide moiety (**Figure 5B**). Subsequently, the newly formed 7-OH internally attacks carbon-7 of the other quinone methide moiety.

Although already synthesized from ethyl ferulate (unpublished results), ferulate tetramers have not been isolated from plant materials to date. Both tetramers isolated and structurally characterized here contain a central 5-5-linkage, but both tetramers also contain the trimer **8** (**Figure 3**) within their structures. Therefore, it is hard to speculate whether these tetramers are formed by coupling of two dimers via a 5-5-linkage or whether a fourth ferulate is coupled to the preformed 5-5/8-*O*-4-coupled dehydrotriferulate. Viable reasons exist for both possibilities. From the biosynthesis of lignin, it is known that 5-5- and 4-*O*-5-linkages in lignin stem from oligomer−oligomer coupling (*30*). Thus, the central 5-5-linkage may be found in both tetramers because similar coupling mechanisms may be true for the formation of ferulate oligomers. From the already mentioned model system, a tetramer with a central 4-*O*-5-linkage was found as a main tetramer, suggesting that similar mechanisms are possible for the coupling of ferulates, at least when low molecular mass ferulates are radically oligomerized. A major difference between ferulate and monolignol dimerization, however, is that ferulate will dehydrodimerize by 5-5-coupling while monolignols do not. Thus, in lignins, 5-5-linkages likely occur exclusively via oligomer−oligomer coupling, whereas they can occur via the monomer with ferulate. Because both isolated tetramers contain trimer **8** in their structure and because this trimer seems to be the predominant trimer in maize bran, it is also conceivable that the tetramers found are formed from trimer **8** by radical coupling of a fourth ferulate. Regarding the 8-5-linkage in compound **4**, the same considerations have to be made as for compound **1**. Again, it is supposed that compound **4** is formed during saponification from a precursor containing an 8-5(cyclic)-coupled unit, the 4-*O*-8/5-5/8-5-(cyclic)-dehydrotetraferulate. Although it is not possible to deduce how many polysaccharide chains are cross-linked by ferulate tetramers, it seems highly unlikely that a ferulate tetramer is formed entirely intramolecularly. The findings of such a diverse array of trimers and now tetramers emphasize the significance of ferulate oligomers as cross-links in the plant cell wall and in cell wall-related food and feed.

In conclusion, the first ferulate dehydrotrimers devoid of 8-*O*-4-coupled units were isolated and identified from maize bran fiber. The 8-8/7-*O*-7-coupling in one of the trimers shows that this kind of linkage is not only restricted to ferulate dimers. In addition, the first ferulate tetramers have been identified in plant material. Both tetramers contain the 5-5/8-*O*-4-coupled trimer, making conclusions about the sequence(s) involved (coupling of two dimers or coupling of a fourth ferulate to a preformed trimer) in the formation of the tetramers difficult. Ferulate dehydrotetramers, however, now need to be recognized as implicating more diverse modes of cell wall polysaccharides cross-linking.

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